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REGULAR PAPER

More nitrogen partition in structural proteins and decreased photosynthetic nitrogen-use efficiency of *Pinus massoniana* under in situ polluted stress

Lan-Lan Guan · Da-Zhi Wen

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Abstract Masson pine (*Pinus massoniana* L.) trees in the Pearl River Delta have shown growth decline since late 1980s, particularly those around industrially polluted regions. As nitrogen is an important nutritional element composing functional proteins, structural proteins and photosynthetic machinery, investigation on nitrogen allocation is helpful to understand nutrient alteration and its regulation mechanism in response to pollution stress. Current year (C) and 1-year old needles (C + 1) of five mature trees were sampled in industrially polluted site and unpolluted natural reserve for bioassay. Needles of declining trees had significantly higher leaf nitrogen per unit area (N_L) but lower photosynthetic capacity (P_{max}), which resulted in lower photosynthetic nitrogen use efficiency (PNUE) than those of healthy trees. Nitrogen fraction to the photosynthetic apparatus in the C and C + 1 needles at polluted site was 27 and 22%, significantly lower than the corresponding healthy needles (48 and 32%). The content of structural proteins was positively correlated with N_L in C and C + 1 needles. Moreover, the C and C + 1 needles of declining trees had about 1.8 times structural protein as those of healthy trees, suggesting that more nitrogen allocation to structural protein are needed for stronger structural defenses under polluted stress. Decreases in PNUE of declining pine trees could be partially explained by increases in structural protein nitrogen.

Keywords Industrial pollution · Pine needles · Nitrogen allocation · Rubisco · Structural protein · Metabolic protein

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Introduction

Anthropogenic activities, such as smelting and petrochemical industry, have caused severe atmospheric pollution in recent decades in China. In 2009, China has emitted 22.14 million tons of SO_2 and 0.523 million tons of industrial dust and one fifth of China's cities are facing severe air pollution situation (Ministry of Environmental Protection of People's Republic of China, 2010). Air pollution is believed to play an important role in forest decline. Among the industrial pollutants emitted directly to the air, ozone, sulfur dioxide, nitrogen oxides, volatile organic chemicals as well as heavy metals-containing industrial dust have been revealed as potential causal agents through direct effects on the foliage and/or more complex disturbances of the forest ecosystems (Weber-Lotfi et al. 2002; Sun et al. 2009).

Masson pine (*Pinus massoniana* L.) is a typical heliophyte tree species naturally and widely growing in South China which plays an important role in constructing subtropical forests in China. Since the beginning of 1990s, South China has become the third largest acid-rain polluted region in the world, following behind Europe and North America (Wang and Ding 1997; Larssen et al. 1999), and

air pollutants have reached very high concentrations in large cities and their vicinities. In Nanshan, the south suburb of Chongqing city in Southwestern China, there are about 2,000 ha of Masson pine forest which has exhibited decline since the beginning of the 1980s, and the damage to Masson pine trees was consistent with an increase in the atmospheric concentrations of SO_2 and fluoride (Bian and Yu 1992). Recent studies have found that this species had declined gradually during the latest decades in Dinghushan Biosphere Reserve and Baiyun Mountain in Guangdong province, by local acid rain and nitrogen deposition (Liu et al. 2007; Lu et al. 2010). SO_2, NO_x and heavy metal toxicity alter the normal growth of conifer tree species by changing amino acid contents and leaf nutrient balance, and both processes involve nitrogen metabolism in plant cells (Thelin et al. 1998; Sun et al. 2009).

Nitrogen is one of the most important nutrition elements composing plant functional proteins, structural proteins and photosynthetic machinery. The photosynthetic capacity of plant leaf is generally well correlated with leaf nitrogen (N) content (Harrison et al. 2009), because a large part of leaf N is invested in the photosynthetic apparatus (Evans and Seemann 1989). N partitioning within a leaf, particularly N allocation to the photosynthetic apparatus is suggested to be a major factor for the variation in photosynthetic nitrogen use efficiency (PNUE) (Hikosaka 2004). Takashima et al. (2004) developed methods for extracting water-soluble and detergent-soluble proteins from leaf material, and assumed that the detergent-insoluble fraction represented the cell wall proteins. The comparison between evergreen and deciduous Quercus species revealed a clear trade-off in N partitioning between photosynthetic proteins (Ribulose-1,5-bisphosphate carboxylase, Rubisco) and cell wall proteins (Takashima et al. 2004). Similar tendency was also obtained for an intraspecific variation in PNUE in *Polygonum cuspidatum* (Onoda et al. 2004). These studies provide data on cell wall N to support the hypothesis put forward by Field and Mooney (1986) suggesting that there may be a trade-off between investing nitrogen in photosynthetic proteins such as Rubisco versus compounds required for longevity. Leaf N partitioning varies within a species. Plants alter their N partitioning within the photosynthetic apparatus depending on growth conditions as the efficient use of N is believed to contribute to fitness of the plant. For example, the proportions of leaf N partitioned into particular N pools are affected by irradiance (Hikosaka 1996), nutrition (Evans and Terashima 1987), timing of germination (Onoda et al. 2004) as well as air pollution (Temple and Riechers 1995). Thus, investigations on N allocation in needles of Masson pine are helpful for us to understand nutrient alteration and its regulation mechanism in response to industrial pollutions.

Despite the well known role of N for multiple critical functions, there has been little research activities on the response of leaf N partition of conifer species under in situ polluted stress. The major goal of this study is to investigate N allocation in Masson pine needles exposed to industrial pollution under field condition. For this purpose, sampling and analyses were conducted on Masson pine trees representing two populations at industrially polluted site and unpolluted Natural Reserve, respectively. We evaluated how leaf N is partitioned in both control and polluted sites at the following two levels: (1) photosynthetic apparatus including Rubisco, bioenergetics (other Calvin cycle enzymes, ATP synthase, and electron carriers) and light-harvesting chlorophyll complex (photosystem I and II); and (2) metabolic and structural compartments.

Materials and methods

Site description and sampling

The study sites are located in Guangzhou (112°57′–114°3′E and 22°26′–23°56′N), the capital and the political and economical center of Guangdong province, China. The area is characterized by subtropical monsoon climate with southwest prevailing wind in summer and north wind in winter. The mean annual temperature was about 21.5°C. The annual precipitation ranged from 1,689.3 to 1,876.5 mm, with a distinct pattern of wet (April through September) and dry (October through March) seasons (Yu and Ng 2007). The polluted forest site is located in Huang Pu industrial district (HP) in eastern of Guangzhou, and the clean site located in remote rural Nan Kun Shan (NKS) Natural Reserve in northeastern of the city. Both of the two sites belong to subtropical monsoon climate characterized by hot and humid summers and cold and dry winters. The lateritic red soil in this region is developed from granite and sand shales (Guangdong Soil Survey Office 1993). HP represents the industrial center and the most polluted area where the electroplating, aluminum and copper refining factories, and the largest petrochemical manufacturing plant were situated. The vigor losses of the Masson pine trees in recent year was visible by observing the crown condition, especially the defoliation and needle chlorosis. In addition, the sulfur and heavy metal concentrations in the needles are much higher in HP than in NKS (Sun et al. 2009). NKS, a natural reserve located away from the centre of Guangzhou, is situated at Longmen county of Huizhou where few industries is situated and low sulfur and heavy metals in pine needles were recorded (Sun et al. 2009). Forest damage and decline at NKS have never been reported. Details on the atmospheric pollution and heavy metal concentration at the two sites are shown in Fig. 1 and Table 1.

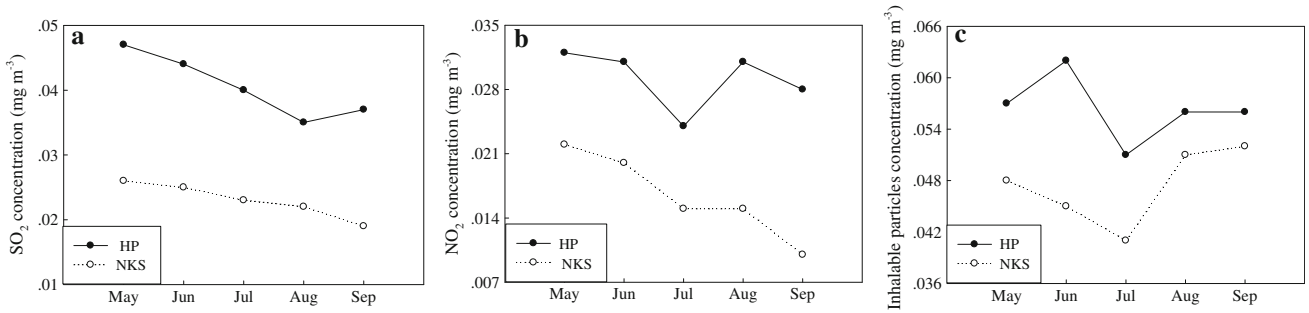

Fig. 1 Air quality nearby the industrial Huang Pu (HP) and remote Nan Kun Shan reserve (NKS) in 2009 (Data from Guangzhou Environmental Protection Bureau and Guangdong Environmental Protection Bureau)

Table 1 Concentrations of heavy metals at industrial HP and remote NKS (Sun et al. 2009)

Heavy metals (mg kg^{-1} dw)	1-year-old needle in HP	1-year-old needle in NKS	Surface soil in HP (0–10 cm)	Surface soil in NKS (0–10 cm)
Cu	7.27 ± 0.96a	2.92 ± 0.22b	3.61 ± 0.84a	2.13 ± 1.31b
Cd	0.34 ± 0.05a	0.04 ± 0.01b	0.04 ± 0.00a	0.03 ± 0.01a
Pb	7.05 ± 1.00a	2.90 ± 0.32b	35.00 ± 3.08a	18.92 ± 4.71b
Zn	52.70 ± 9.84a	42.55 ± 4.97b	101.63 ± 3.66a	89.66 ± 4.95b
Cr	2.33 ± 0.60a	0.62 ± 0.40b	11.86 ± 2.37b	18.17 ± 1.52a
Ni	1.02 ± 0.21a	0.47 ± 0.10b	1.30 ± 0.64a	0.76 ± 0.48a

Different lowercase letters indicate significant differences ($P < 0.05$) in needles or surface soil between the two sites

Five mature trees at each site were chosen for sampling and measurements. Diameter at breast height (DBH) of selected trees ranged from 18 to 25 cm with a mean of 20 cm. These trees are of similar age (about 30 years old) without visible damages and estimated to be between 7 and 10 m in height, except that the pine trees at polluted HP were relatively smaller in DBH, height, and canopy length than those at NKS. In September 2009, needles of the current year needles (C, year 2009) and 1-year old (C + 1, year 2008) were selected from outer branches of the middle canopy of each tree for photosynthetic measurements and then collected for bioassay.

Gas exchange measurement

Photosynthesis measurements were made for the current year and 1-year old needles of five mature pine trees at each site with an open gas exchange system (Li-6400; Li Cor, Lincoln, NE, USA) (Warren et al. 2003) in September 2009. Branches of 80–120 cm in length and 12–15 mm in diameter were sampled from the middle canopy using pruning shears and the branch bottom was immediately putted into water-containing plastic bottle. Previous studies (Warren et al. 2003; St Clair et al. 2005) and our preliminary experiment showed that excision had no systematic effect on the measured rates of photosynthesis or stomatal conductance during the measuring period. Maximum photosynthetic rates (P_{max}) were measured at photosynthetic photon flux density of 1,500 μmol $m^{-2}s^{-1}$ generated by the 6400-40 LED light source in the mid morning at CO_2 concentration of 380 μmol mol^{-1}. Conditions in the leaf chamber were controlled (vapour pressure deficit at leaf surface = 1.0 + 0.2 kPa, leaf temperature = 30°C). Photosynthetic nitrogen-use efficiency (PNUE) was calculated as P_{max}/N_L (μmol CO_2 mol^{-1} N s^{-1}) (Pons et al. 1994). When measuring CO_2 response curves, photosynthetic rates were determined at various air CO_2 partial pressures at photosynthetic photon flux density of 1,500 and 200 μmol $m^{-2}s^{-1}$ generated by the 6400-40 LED light source, respectively. Eight measurements were taken (partial pressures of ambient CO_2 (Ca) were 50, 100, 200, 350, 500, 700, 900, 1,100 μmol mol^{-1}) for each curve. Measurements began at Ca of 350 μmol mol^{-1}, then Ca was decreased as the order of 350, 200, 100, and 50 μmol mol^{-1}; after this sequence was completed, Ca was returned to 350 μmol mol^{-1}, then increased sequentially and stepwise to 1,100 μmol mol^{-1}, to develop the CO_2 response curves. Upon completion of a curve, needles were dissected out of the chamber, their areas were determined and photosynthetic rates were calculated on a projected area basis.

According to Brooks and Farquhar (1985), the response curves were linear initially and therefore a linear regression was fitted through the data. At the point where the two response curves intersected each other, which were measured at high (1,500 μmol $m^{-2}s^{-1}$) and low (200 μmol $m^{-2}s^{-1}$) irradiances, the ordinate was the rate of non-photorespiratory CO_2 evolution in the light (R_d) and

the abscissa was CO_2 compensation point in the absence of R_d (Γ_*).

Leaf nitrogen and chlorophyll content

The projected surface area was measured by using LI-3000 Portable Area Meter (LI-COR, Biosciences, Lincoln, NE, USA) (Temple and Riechers 1995). Leaf mass per area (LMA), leaf nitrogen per unit area (N_L) and chlorophyll (Chl) content in all samples were determined with 5 replicates of separate trees at each site. LMA was calculated as the mass/area ratio of leaf samples after oven dried at 60°C for at least 72 h. N_L was determined in the same dry matter with kjeldahl method. Chlorophyll was extracted from 0.1 g fresh needle with 10 ml 80% acetone and the extracts were measured by using a spectrophotometer (Unico, UV3802) at 663, 646 and 470 nm respectively, according to Lin et al. (1984). The other needles were snap-frozen in liquid nitrogen and stored at −80°C.

Determination of protein content

The Rubisco concentration was determined according to Makino et al. (1986). Frozen needles ($n = 6$) were powdered in liquid nitrogen in a mortar with a pestle and homogenized in 3 ml of 62.5 mM Tris–HCl buffer (pH 6.8) containing 2% (w/v) SDS, 5% (w/v) glycerin, 2 mM EDTA, 1 mM PMSF, 3% (v/v) ME and 10% (w/w) insoluble polyvinylpolypyrrolidone (PVPP). The homogenate was centrifuged at 12,000*g*, 4°C for 30 min, and the supernatant was applied to sodium dodecyl sulfate-polyachrylamide gel electrophoresis (SDS-PAGE). The concentration of separation gel and stacking gel was 12% (w/v) and 5% (w/v), respectively. Then the gel was stained with Coomassie Brilliant Blue R-250. The bands of the large and small subunit of Rubisco was cut and extracted with formamide for spectrophotometric determination of Rubisco.

Metabolic and structural proteins were separated by solubility in SDS (Yasumura et al. 2006; Takashima et al. 2004) with minor modification. Frozen leaf pieces were powdered in liquid nitrogen in a mortar with a pestle and homogenized in 100 mM phosphate buffer (pH 7.0) containing 3% (w/v) SDS and 10% (w/w) insoluble PVPP. The mixture was heated at 90°C for 5 min, and then centrifuged at 4,500*g* for 10 min. Thereafter, the phosphate buffer with 3% (w/v) SDS was added to the pellet, heated and centrifuged again. This procedure was repeated three times. All the supernatants through this process were collected as metabolic proteins. The final pellet represented the structural proteins. Metabolic and structural proteins were determined with ninhydrin after hydrolysis to amino acids with 0.316 mmol Ba $(OH)_2$ and purified water in an autoclave (120°C, 0.10 MPa) for 30 min. Bovine serum albumin was used as the protein standard.

Nitrogen and protein calculation

The in vivo performances of Rubisco (maximum rate of carboxylation, V_{cmax}) and the maximum rate of electron transport in chloroplasts (J_{max}) were determined from CO_2 response curve of photosynthesis according to a biochemical model of photosynthesis (de Pury and Farquhar 1997). The kinetic constants of Rubisco (K_c and K_o, the Michaelis–Menten constants for CO_2 and O_2, respectively) were adopted from von Caemmerer et al. (1994). K_c was assumed to be 40.4 Pa and K_o was 24.8×10^3 Pa. In vivo specific activity of Rubisco estimated as V_{cmax}/Rubisco content per unit area. Nitrogen in Rubisco (P_R) was calculated assuming that nitrogen concentration in Rubisco is 16% (Hikosaka and Terashima 1995). Nitrogen in bioenergetics (P_B) was estimated from gas exchange characteristics. Nitrogen in bioenergetics is assumed proportional to J_{max}, where the ratio of J_{max} to the cytochrome f content is 156 mmol mol^{-1} s^{-1} and nitrogen in bioenergetics per unit cytochrome f is 8.06 mol $mmol^{-1}$ (Niinemets et al. 1998). Nitrogen in light harvesting complex (P_L) is assumed proportional to chlorophyll concentration, where the parameter of nitrogen invested in thylakoids participating in light harvesting is 5.87 mmol Chl $(g\ N)^{-1}$ (Hikosaka and Terashima 1995).

The amount of N partitioned into protein fractions was estimated based on the assumption that all leaf proteins have an N concentration of 16% (Field and Mooney 1986). The amount of 'other protein N' was estimated as the difference between nitrogen in the metabolic protein and in the photosynthetic apparatus ($P_R + P_L + P_B$). 'Other nitrogen' was calculated as the total leaf nitrogen minus nitrogen in protein (metabolic and structural proteins).

Statistical analyses

SPSS 15.0 (SPSS, USA) for Windows was used for the data analysis. Data were presented as mean ± standard deviations (SD). Paired *t* test was performed to compare the difference of a parameter between two sites and that between C and C + 1 needles within a site. Effects with probabilities of $F > P < 0.05$ were considered to be significant. Correlation analyses between two parameters were performed by Pearson test ($P < 0.05$).

Results

The C + 1 needles had higher LMA than C needles in HP and NKS, whereas there was no significant differences in

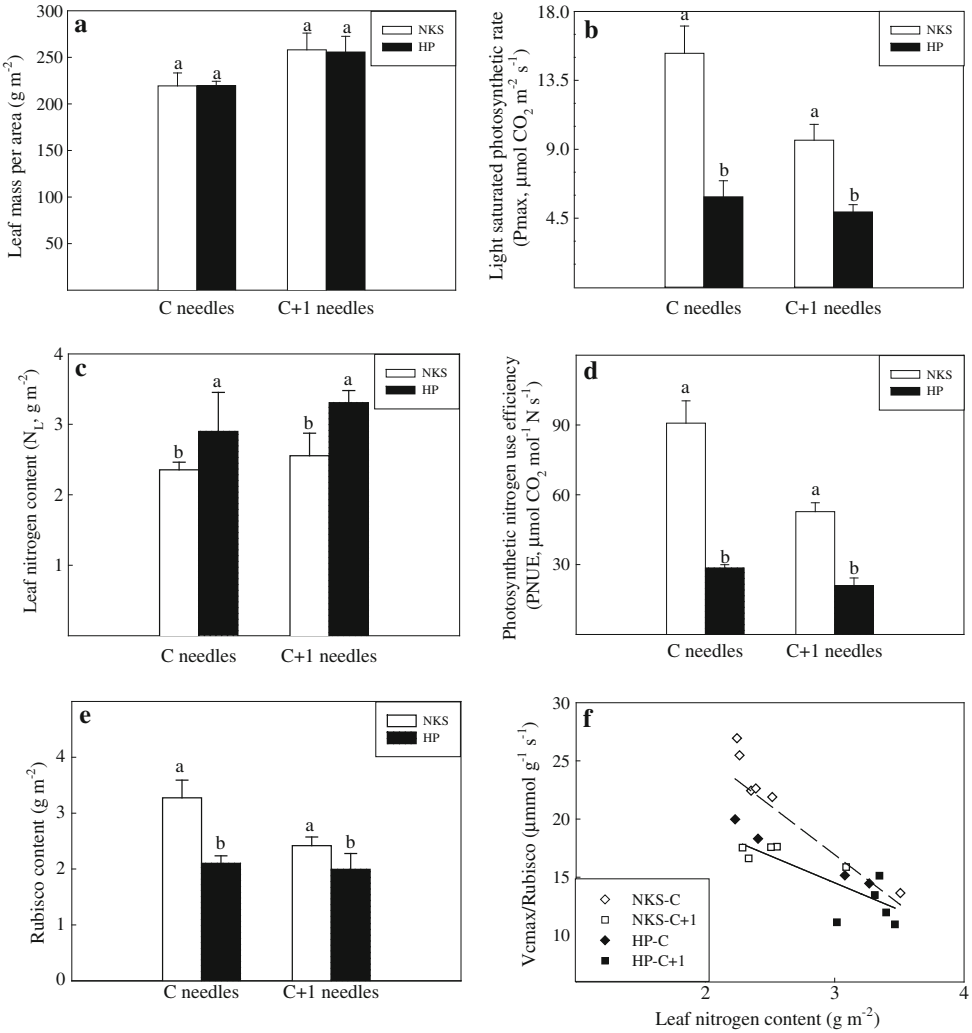

Fig. 2 LMA (**a**), light saturated photosynthetic rate (**b**), needle nitrogen concentration (**c**), photosynthetic nitrogen use efficiency (**d**) and Rubisco content (**e**) and in vivo specific activity of Rubisco (**f**) in the current year (C) needles and one-year-old (C + 1) needles at remote NKS and industrial HP sites. *Data* with *different letters* showed statistical differences at $P < 0.05$

the same age needles between the two sites (Fig. 2a). Light-saturated photosynthetic rates (P_{max}) in the C and C + 1 needles of Masson pine trees in polluted HP site were significantly lower than those in unpolluted NKS, whereas N_L was higher in HP site (Fig. 2b, c), which consequently resulted in a significantly higher photosynthetic nitrogen use efficiency (PNUE) of the trees at NKS site (Fig. 2d). In addition to the effects on P_{max} and total N, pollution significantly affected N contents in photosynthetic apparatus that the Rubisco content at NKS was 55.8% higher (C needles) and 21.3% higher (C + 1 needles) than the corresponding needles at HP site. Besides, significant effects of needle age on the Rubisco content at NKS was also observed as that was 35% higher in C needles than in C + 1 needles. However, this age effect was not significant for trees at polluted HP (Fig. 2e). Pmax was positively correlated with the Rubisco content in both C ($r = 0.923$, $P < 0.05$) and C + 1 needles ($r = 0.751$, $P < 0.05$). Moreover, in vivo specific activity of Rubisco (V_{cmax}/Rubisco) in the needles was significantly greater at NKS than that at HP (Fig. 2f). V_{cmax}/Rubisco was significantly negatively correlated with N_L in both C ($r = 0.900$, $P < 0.05$) and C + 1 ($r = 0.794$, $P < 0.05$) (Fig. 2f).

Needle N partitioning to photosynthetic proteins is shown in Fig. 3. The partition coefficients for N in Rubisco (P_R), in bioenergetics (P_B), and in thylakoid light-harvesting components (P_L) were significantly higher in both the C and C + 1 needles from NKS than those from HP site. Moreover, P_R and P_B in the C needles in NKS site were almost twice as those in HP site. However, P_L was less altered between the two sampling sites, only 19% decrement was found in HP needle samples.

As SDS removes soluble and membrane-associated proteins, the SDS-insoluble fraction of protein represented cell wall proteins that are tightly bound to cell walls (Takashima et al. 2004). In the present study, we observed that the metabolic protein content in C needles was similar as that in C + 1 needles either at NKS or at HP sites, and there was no significant difference in metabolic protein content of C needles between the two sites. However,

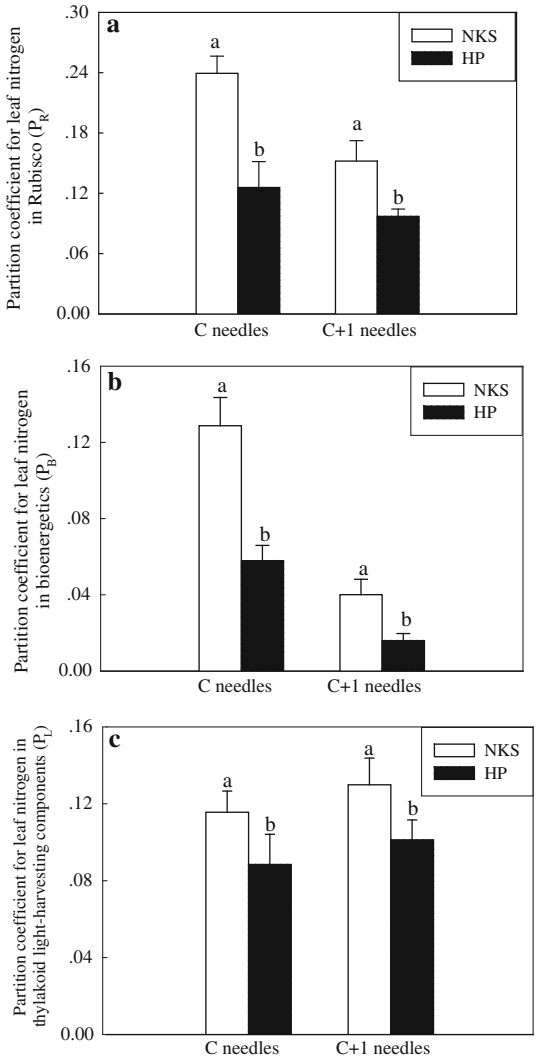

Fig. 3 Needle nitrogen partitioning coefficient to Rubisco (P_R), bioenergetics (P_B), and the thylakoid light-harvesting components (P_L) in the current year (C needles) and one-year-old (C + 1 needles) at remote NKS and industrial HP sites. *Data* with *different letters* showed statistical differences at $P < 0.05$

structural protein in the C needles at HP site showed nearly 1.8 times as that of the same aged needles at NKS site. Both metabolic and structural protein contents in the C + 1 needles from NKS site were significantly lower than those from HP site (Table 2). In general, the metabolic protein content was relatively stable against, and not significantly correlated with the different N_L of the pine trees at the two sites. Structural protein content was positively correlated with N_L in C needles ($r = 0.831$, $P < 0.05$) and C + 1 needles ($r = 0.855$, $P < 0.05$) across different growth conditions (Fig. 4b).

Figure 5 summarizes nitrogen allocation in the C and C + 1 needles at the two sites. Metabolic protein accounted for 57–71% of total leaf N in all needles. N fraction to the photosynthetic apparatus in the C and C + 1 needles ($P_R + P_L + P_B$) at HP site was 27 and 22%, significantly lower than the corresponding needles (48 and 32%, respectively) at NKS site. In contrast, the C and C + 1 needles at NKS site displayed lower N partitioning fractions to other protein N than those at HP site. Similar to allocation (6–13%) in SDS-insoluble proteins reported by Takashima et al. (2004), structural proteins explained only a small proportion (7–13%) of N_L in all the samples. The proportion of N allocation to structural proteins was higher in C and C + 1 needles at HP than the corresponding needles at NKS. The higher needle N content at HP site was not efficiently contributed to photosynthetic proteins but to structural and undetectable proteins.

Discussion

Partitioning of leaf N in photosynthesis

Air pollution and their derivates (acid and nitrogen deposition) necrotized plant tissue and resulted in subtropical forest tree decline by altering leaf free amino acid content, photosynthetic rate, and whole plant biomass accumulation (Liu et al. 2009; Lu et al. 2010). Tree-ring study also revealed growth decline of Masson pine trees at HP site since 1980s (Kuang et al. 2008). In the present study, lowered P_{max} at polluted HP site can be explained by the adverse effects of multiple pollutants including SO_2, NO_x. It is quite evident in the researches on physiological and biochemical processes of plants, for example, air pollution may deteriorate chloroplast pigments and alter the chloroplast ultrastructure, thus lower the PSII efficiency and relative electron transport rate (Liu et al. 2009). Besides air pollutants, heavy metals are harmful substances in industrial emissions which are responsible for forest die back. Masson pines growing in HP site had remarkably higher Cu, Cd, Pb, Zn, Cr and Ni contents in C + 1 needles than those in NKS site as shown in Table 2 (Sun et al. 2009). Previous studies also showed that heavy metal toxicity in higher plants is associated with growth inhibition, enzyme activities alteration and photosynthesis reduction (Verma and Dubey 2003). Thus, the toxic heavy metals from industrial dust might be another factor contributing to the reduced photosynthetic capacity.

Generally, P_{max} is strongly correlated with the leaf N content per unit area (N_L). Higher N content is always associated with higher photosynthetic rate because large amount of leaf organic nitrogen is located in photosynthetic machinery (Poorter and Evans 1998). However, this relationship was not true in the present investigation because the declining Masson pine trees at HP site had higher N_L but lower P_{max} than the healthy ones at NKS site. Here, the relatively higher NO_x pollution is thought to be one of the

Table 2 The content of nitrogen allocated to metabolic protein, structural protein, Other protein N, and Other N in the C (NKS-C, HP-C) and C + 1 (NKS-C + 1, HP-C + 1) needles at remote NKS and industrial HP sites

	NKS-C	HP-C	NKS-C + 1	HP-C + 1
N in metabolic protein (g m^{-2})	1.75 ± 0.16a	1.80 ± 0.12a	1.65 ± 0.12b	1.90 ± 0.15a
N in structural protein (g m^{-2})	0.18 ± 0.04b	0.31 ± 0.04a	0.22 ± 0.02b	0.42 ± 0.03a
Other protein N (g m^{-2})	0.54 ± 0.03b	0.95 ± 0.18a	0.82 ± 0.10b	1.20 ± 0.06a
Other N (g m^{-2})	0.51 ± 0.02b	0.84 ± 0.16a	0.70 ± 0.09b	0.97 ± 0.05a

Different lowercase letters indicate significant differences ($P < 0.05$) in needles of the same age between the two sites

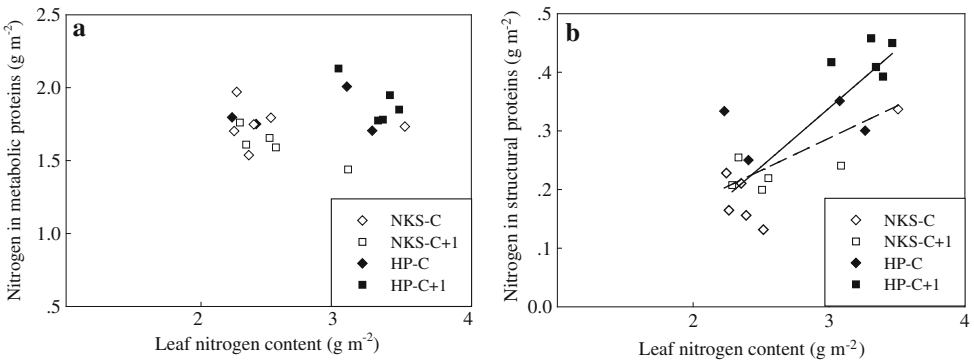

Fig. 4 Correlations of nitrogen in metabolic proteins and nitrogen in structural proteins with the C and C + 1 needle nitrogen concentrations at remote NKS and industrial HP sites, respectively

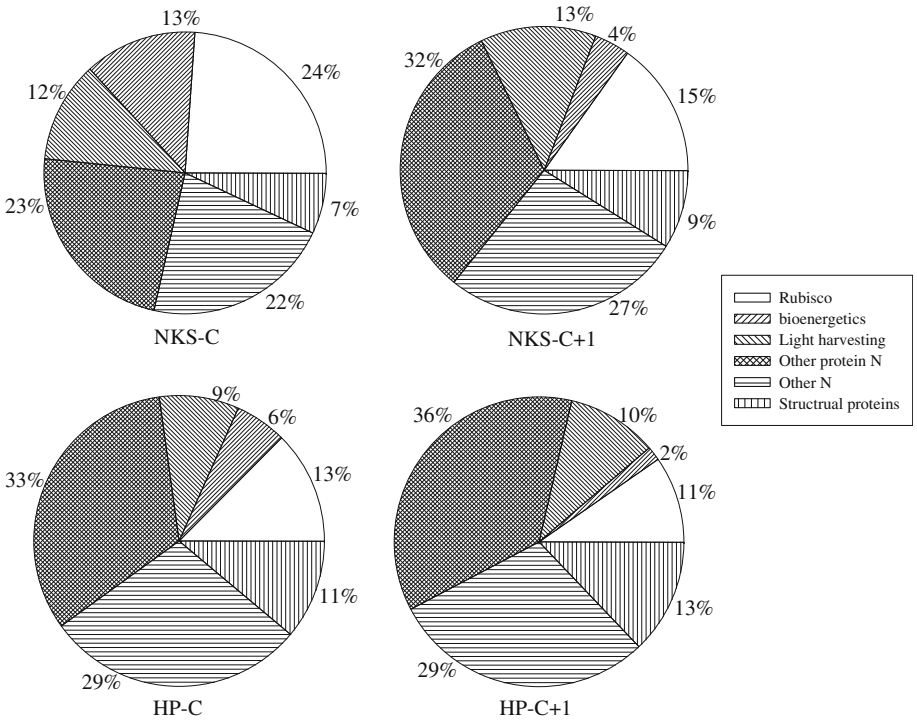

Fig. 5 Nitrogen partitioning fractions in the C (NKS-C, HP-C) and C + 1 (NKS-C + 1, HP-C + 1) at remote NKS (**a**) and industrial HP (**b**) sites

sources of the additional N in Masson pine needles at polluted site. It was reported that needle $\delta^{15}N$ (Kuang et al. 2010) and tree ring $\delta^{15}N$ (Sun et al. 2010) at the NKS were much higher than those at polluted site, which was proved to indicate the severe N deposition at polluted site. High foliar N concentrations are usually associated with high rates of atmospheric N deposition, although amounts of foliar N can also be excessive in areas with low N deposition rates. Another possible source of N enrichment in current year needles at HP site may be the increased rates of protein turnover resulted from increasing proteolysis activity in prematurely senescing foliage of the declining

trees due to air pollutants (Manderscheid et al. 1992), which would increase the availability of N for reallocation to actively growing parts of the plant (Temple and Riechers 1995).

Taken together, higher N_L and lower P_{max} of declining trees at polluted HP site resulted in decreased PNUE compared with healthy trees at NKS site. In our results, this lowered PNUE was associated with a smaller allocation of nitrogen to the photosynthetic apparatus (Fig. 3), which coincided with former observations across species (Westbeek et al. 1999; Ripullone et al. 2003). Since net photosynthetic rate of Masson pine was mainly determined by the amount of Rubisco in the needles, the reduction of PNUE is mostly associated with the decrease in needle N allocation to Rubisco (Yamaguchi et al. 2007). N allocation to the photosynthetic apparatus is suggested to be a major factor for the interspecific (Hikosaka 2004; Hikosaka and Shigeno 2009) and intraspecific (Onoda et al. 2004) variations in PNUE, thus the degree of reduction in the amount of Rubisco between the two sites was dependant on the reduction in the availability of N to Rubisco synthesis in the leaves.

Partitioning of leaf nitrogen in metabolic protein

Most perennial plants can store N in a variety of forms in plants but in foliage it is most often stored as inorganic N (nitrate or ammonium), free amino acids or proteins (Millard 1988). However, the protein is the largest store-owing simply to the greater content of protein N in foliage (Warren et al. 2003). Here, we divided needle proteins of Masson pine trees into two fractions as metabolic and structural proteins, and the metabolic proteins include soluble enzymes in stroma, cytosol and the membrane-associated proteins such as thylakoid components (Evans and Seemann 1989; Takashima et al. 2004). Our result showed that the allocation proportion of metabolic proteins in C and C + 1 needles at HP was lower than the same age needles at NKS. However, to compare the real content, metabolic protein in needles at HP site was equivalent (C needles) or even slightly higher (C + 1 needles) than that of the corresponding needles in NKS. Therefore, we deduce that metabolic proteins could be a possible N pool in the needles with great N_L.

Among the photosynthetic proteins, Rubisco is the key enzyme acting in the carbon assimilation and Calvin cycle and occupies about half of photosynthetic nitrogen content (Evans and Seemann 1989). The relationship between Rubisco content and N_L is extremely complicated. In a study with *Pinus sylvestris*, Warren et al. (2003) found N_L in needles increased significantly with greater N supply, and Rubisco-N content in needle was positively related to N_L. The estimated in vivo specific activity of Rubisco was negatively related to N_L. Hence, they concluded Rubisco content was in excess of the amount required for photosynthesis and this excess was positively related to N_L, which support that with increasing N_L, Rubisco functions increasingly as a storage protein in addition to its catalytic functions. In foliage of apple (Cheng and Fuchigami 2000) and Douglas-fir seedlings (Manter et al. 2005), Rubisco activation decreased with increasing N_L, which also supported that Rubisco could serve as a storage protein in leaves with a high N content. Without exceptions, the above researches focused on the healthy plant individuals growing in relative clean environment. In our study, higher N_L at HP was probably induced by nitrogen deposition from air pollution rather than nutrition supply. In such condition, V_{cmax}/Rubisco was significantly negatively related to N_L in C and C + 1 needles in accordance with previous findings. However, with higher N_L, the Rubisco content of the C and C + 1 needles from the trees at HP site were significantly lowered than that of corresponding needles at NKS. P_R decreased with increasing N_L at polluted HP. The reason is that the degradation of photosynthetic apparatus under air pollution definitely reduces Rubisco content in leaf (Yasumura et al. 2006). We also found that Rubisco content seemed to have been degraded more rapidly than other metabolic proteins due to air pollutants. Therefore, under air pollution, Rubisco could not serve as a storage protein in leaves as N_L increases.

In contrast with P_R, P_L and P_B, the C and C + 1 needles at HP site displayed higher N partitioning fractions to Other protein N than those at NKS site. As known, air pollution could cause serious oxidative damage such as DNA variation, lipid peroxidation, chlorophyll, protein degradation and photosynthesis reductions as revealed before (Weber-Lotfi et al. 2002; Liu et al. 2009). To cope with oxidative stress, plant develops a series of antioxidant enzyme and antioxidant pigment so as to safely degenerate free radicals in plant tissue (Liu et al. 2007). In the present study, the higher "Other protein N" content under air pollution might be attributed to the elevated antioxidant enzyme content and activity, such as peroxidase and catalase, in Masson pine needles (unpublished data). Hence, whether and how the metabolic proteins could store N in higher-N_L needles under air pollution deserves further research.

Partitioning of leaf nitrogen in structural protein

In the present study, with the increasing N_L, allocation of N to Rubisco and total metabolic proteins decreased in both C and C + 1 needles at HP. Meanwhile, a remarkable elevation of structural proteins was found in leaf samples at HP. It is surprising that needles of declining trees invested more N into the structural proteins which is comparable

with that invested into Rubisco. Moreover, structural protein content was positively correlated with N_L. We could deduce that Masson pine trees store the "excess" N in structural proteins under air pollution.

Takashima et al. (2004) found leaf N allocation to SDS-insoluble proteins increased with increasing LMA. However, in our study, leaf N allocation to SDS-insoluble proteins at polluted site increased while LMA was similar to that at unpolluted site, suggesting that cell wall proteins may not be the only source leading to the increase of SDS-insoluble proteins. On the other hand, the cell death determination by using Evans Blue dye as a marker found that air pollution at HP sites induced cell death partially in needles of Masson pine (unpublished data), by which the leaf mass could be affected. Moreover, part of phenolics in the vacuole might diffuse into the cytolist and bind to proteins through hydrogen bond and ionic bond in polluted needles immediately after the lysis of plant cells, and the polyphenol–protein complex is more hydrophobic and susceptible to protein aggregation and precipitation (Pierpoint 2004). Hence, it is possible that a few changes of SDS-insoluble proteins in polluted needles might be resulted from these precipitated polyphenol-proteins.

Investigations on naturally growing plants have confirmed the trade-off in N allocations between N allocation to photosynthetic proteins and to structural proteins. Onoda et al. (2004) investigated the intraspecific variation in PNUE in Polygonum cuspidatum with different germination time but similar defoliation time. They showed that early germinators invested more N in structural proteins at the expense of allocation to Rubisco, and had a lower PNUE. Also, evergreen Quercus species increased N partitioning to structural proteins compared with deciduous Quercus species, which was accompanied by a reduced investment in photosynthetic proteins. And this trade-off may result in lower PNUE (Takashima et al. 2004). In a study of an invasive plant in China and India (*Ageratina adenophora*), Feng et al. (2009) found this plant increased N allocation to photosynthesis (growth) and reduced allocation to cell walls. However, the striking trade-off between N allocation to the photosynthetic proteins and structural proteins is unlikely to hold as a general rule. A study dealing with two groups of plants that were consisted of two perennial evergreen species and seven *Eucalyptus* species, revealed that no trade-off between N associated with cell wall and N allocated to Rubisco (Harrison et al. 2009). Hence, they suggested that variation in cell wall N could not account for the variation in PNUE. Another study was focusing on the seasonal changes in N partitioning (Yasumura et al. 2006). They found once *Lindera umbellata* leaves were fully expanded, leaf N partitioning to Rubisco was relatively stable whereas that to structural protein was increased until death. During the leaf lifespan, there was no trade-off in N partitioning between Rubisco and structural proteins. Our study showed that nitrogen allocation to the structural proteins increased while less nitrogen was allocated to photosynthetic proteins for the declining C and C + 1 needles at HP, suggesting that there is a competitive trade-off between N allocation to photosynthetic proteins and to structural proteins. The difference in nitrogen allocation to the photosynthetic apparatus at both sites was partially counterbalanced by greater allocation of nitrogen to structural proteins. More nitrogen partition in structural proteins can partially explains the decreases in PNUE of declining pine trees under industrial pollution.

It is appealing to think why the declining trees with high N_L had a greater amount of structural proteins at the expense of decreasing PNUE. Although the exact underlying function of structural proteins remains to be clarified (Onoda et al. 2008), some studies have suggested that the greater structural proteins contributes to mechanical toughness of leaves which led to stronger structural defenses (Showalter 1993), and some others showed that mechanical protection was important for maintaining leaves for a longer growing period (Takashima et al. 2004). Leaf toughness is an important leaf structural trait contributing to plant resistance against insects (Ossipov et al. 2001). Toughness could affect herbivore performance through influencing insect life history parameters or altering the ratio of macronutrients assimilated from the gut of the herbivores and increasing metabolic costs of insects (Clissold et al. 2009). A Masson pine forest survey implemented in the acid rain region in south China has revealed the insect defoliation could be one of the contributing factors causing the forest decline at polluted sites (Wang et al. 2007). Hence, the leaf defense ability against insects is critical for Masson pines growing in the air polluted areas. The evidence that structural protein content was positively correlated with N_L in Masson pine trees suggested that the increased N_L in declining needles under polluted stress contributed the higher content of structural proteins. The increasing nitrogen in declining Masson pine needles did not bring high $P_{max,}$ but needles allocated more N for leaf toughness when Masson pine were exposed to the air pollution.

Conclusions

We determined photosynthetic responses and nitrogen partition of Masson pine needles from stands at industrial polluted and unpolluted sites, and draw the following conclusions. First, N content was elevated in needles from polluted site, which might be partially caused by N deposition, whereas PNUE together with the reduced

photosynthetic activities was largely decreased. Secondly, there is clearly a trade-off between N allocation to the photosynthetic apparatus particularly Rubisco and structural protein in Masson pine needles under polluted stress—the declining trees with higher N_L allocated more nitrogen for leaf toughness at the expense of decreased PNUE than the healthy trees.

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